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Oxidative status of platelets in normal and thalassemic blood

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Summary
Chronic platelet activation may be involved in thromboembolic complications, a leading cause of morbidity and mortality in β-thalassemia. Oxidative stress, with the generation of reactive oxygen species (ROS), is suspected to play a role in the pathophysiology of thalassemia and cardiovascular disorders. In the present study, we adapted flow cytometric techniques to measure oxidative state markers, ROS generation and reduced glutathione (GSH) content in platelets. Our results show that platelets obtained from β-thalassemic patients contain higher ROS and lower GSH levels than do platelets from normal donors, indicating a state of oxidative stress. In the absence of any known inherent abnormality in thalassemia platelets, this may be attributed to continuous exposure to oxidative insults from extra-platelet sources. We found that exposure of platelets to oxidants such as hydrogen peroxide and tert-butylhydroperoxide or to the platelet activators thrombin, calcium ionophore or phorbol myristate acetate stimulated the platelets' oxidative stress. This was also increased by plasma of thalassemia patients, and decreased following treatment of the plasma with the iron-chelator Desferoxamin. Iron and hemin, the levels of which are augmented in plasma of thalassemia patients, stimulated the platelets' oxidative stress. The oxidative status of the platelets was also affected by red blood cells (RBC); it was higher in normal platelets incubated with thalassemic RBC than with normal RBC. Normal RBC stimulated with hydrogen peroxide had a greater effect on platelets than did unstimulated RBC. The platelets' oxidative stress was ameliorated by antioxidants such as N-acetyl-L-cysteine and vitamin C. Our findings indicate that in thalassemia, platelets undergo a state of oxidative stress, leading to their activation and potentially to thromboembolic consequences, and suggest that this hypercoagulable state might be treated with antioxidants.

Keywords
Thalassemia, platelets, free radicals, glutathione, flow cytometry

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Introduction

Thromboembolic complications are an important cause of morbidity and mortality in β-thalassemia (1). As platelets play a key role in hemostasis and thrombosis, many studies examined whether platelet function is altered in this disease. The chronic platelet activation, which is characteristic of thalassemia, was demonstrated by the presence of an increased fraction of platelets carrying the activation markers CD62P (P-selectin) and CD63 (2, 3) and the externalization of phosphatidylserine (4). In addition, morphological changes in platelets from thalassemia patients, increased spontaneous whole blood platelet aggregation and decreased platelet life span, as well as elevated plasma platelet factor 3 and augmented urinary excretion of T_xA_2 metabolites were also reported (1, 3). The mechanism of potentiated platelet activation in β-thalassemia is not clear, but it is possible that it is associated with RBC abnormalities and components present in the patient's plasma (5).

Oxidative stress, with the generation of different reactive oxygen species (ROS), plays a significant role in the pathophy-

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siology of various diseases, including thalassemia and cardiovascular disorders (6). Previous investigations demonstrated that ROS profoundly affect platelet function and promote platelet activation (7, 8). Furthermore, many studies have shown that the platelets themselves have the ability to produce low levels of ROS, which may be increased by various platelet activators such as thrombin (9-11).

The oxidative status represents the balance between oxidative and antioxidative systems. The antioxidant system includes enzymes such as glutathione peroxidase, superoxide dismutase and catalase (12). Reduced glutathione (GSH) is the major intracellular antioxidant and is considered an important scavenger of ROS. Aside from its role in maintaining the redox potential within the cell, it is also a key component of the enzymatic antioxidant system (13-16).

The oxidative status of cells, including that of platelets, is difficult to determine because of the technical difficulties involved in measuring oxidative radicals and the fact that even slight contamination by other blood cells, such as neutrophils, may bias the results (14, 17). In the present study, we utilized flow cytometry measurements of oxidative state markers, ROS generation and GSH content, to compare platelets from normal donors and patients with β-thalassemia. Our results indicate that thalassemia platelets contain higher levels of ROS and lower levels of GSH than do normal platelets and raise the possibility of using antioxidants as prophylactic treatment for thromboembolic complications.

Materials and methods

Platelet and RBC isolation

Peripheral blood of normal and thalassemic donors was collected in tubes containing 0.129 M trisodium citrate (Becton Dickinson, Plymouth, UK). None of the normal subjects had been on medication for at least 4 weeks prior to the day of measurement. The patients had different mutations, exhibited various levels of clinical severity and had undergone diverse treatment regimes, although most of the patients (>85%) suffered from β-thalassemia major, were polytransfused and on iron-chelation therapy. In transfused patients, blood was obtained prior to blood transfusion, i.e., at least 3 weeks following the previous transfusion. Informed consent was obtained in all cases. The blood was centrifuged for 12 min. at 800 rpm and the platelet-rich plasma (PRP) was collected from the upper layer, while the RBC were collected from the lower layer. The plasma and platelets were separated by centrifugation of the PRP for 3 min. at 1200 rpm. The platelets were washed twice and resuspended in Ca^{++}– and Mg^{++}–free Dulbecco's phosphate-buffered-saline (PBS) (Biological Industries, Kibbutz Beit-HaEmek, Israel) and used within 2 hrs of blood withdrawal.

ROS and GSH assays

To measure ROS generation, platelets were incubated with 0.4 mM (final concentration) 2'-7'-dichlorofluorescin diacetate (DCF) (Sigma, St. Louis, MO), dissolved in methanol (Bio Lab, Jerusalem, Israel). After incubation for 15 min at 37°C in a humidified atmosphere of 5% CO_2 in air, the platelets were washed twice and resuspended in PBS. To measure the GSH content, platelet concentrates were incubated for 3 min. at room temperature with 40 μM (final concentration) [1-(4-chloromercuryphenyl-azo-2-naphthol)] (mercury orange) (Sigma). A 100 μM stock solution of mercury orange was made up in acetone and stored at 4°C.

Oxidants and antioxidants

The following compounds were used to modulate the platelets' oxidative status: Hydrogen peroxide (H_2O_2) (Biological Industries), hemin, [in the form of heme arginate (Leiras Oy, Turku, Finland) or heme chloride], iron (in the form of ferrous ammonium sulfate), thrombin, calcium ionophore A23187 and phorbol 12-myristate 13-acetate (PMA), N-Acetyl L-cystein (NAC), N-ethylmaleimide (NEM), diethylmaleate (DEM), desferroxamin, t-butylhydroperoxide (BHP) (all purchased from Sigma) and vitamin C (Dolder Ltd, Basel, Switzerland).

Flow cytometry

Platelets, treated as described above, were analyzed with the aid of a Fluorescence Activated Cell Sorter (FACS-calibur, Becton-Dickinson, Immunofluorometry Systems, Mountain View, CA). The platelets were passed at a rate of about 1,000/ sec, using saline as the sheath fluid. A 488-nm argon laser beam was used for excitation. The FACS was calibrated and set using CaliBRITE^{TM}-3 beads (Becton-Dickinson). To exclude non-platelets from analysis, a two-parameter dot-plot of the side light scatter (SSC) and forward light scatter (FSC) of the population was first analyzed. A gate was set to include only platelets and to exclude any RBC or white blood cells (WBC), that might be present (19). Platelets were distinguished by their characteristic light scatter (log scale) and binding of phycoerythrin (PE)-conjugated anti-CD41 monoclonal antibody (IQ Products, Groningen, The Netherlands). Platelets stained with DCF were detected by the FL-1 PMT, and those stained with mercury orange or anti-CD41 – by the FL-2 PMT, using log amplification. The Mean Fluorescence Channel (MFC) was calculated for DCF and mercury orange with FACS-equipped CellQuest^{R} software. For each assay, unstained platelets, both treated and untreated, served as controls; their MFC was in the range of 3-7.

Statistical analyses

The results are expressed as the mean ± standard deviation and compared by the two-sample Student's t-test for differences in means.

Results

ROS generation and GSH levels were measured in normal and thalassemia platelets, using DCF and mercury orange, respectively, as described in *materials and methods*. Platelets were gated on the basis of size and granularity (FSC and SSC) (Fig. 1A). The identity of the gated platelets was verified by immunofluorescence staining for CD41, a platelet specific antigen (Fig. 1B).

Histograms of platelets from representative normal and thalassemic samples stained with DCF and mercury orange are shown in Figures 1C and D. The Mean Fluorescence Channel (MFC) of the DCF- stained platelets of normal and thalassemia samples was 61 and 681, respectively, and the MFC of mercury orange was 294 and 29, respectively. The results suggest a higher ROS generation and a lower GSH content in the platelets of thalassemia patients than in platelets of normal controls.

Figure 2 shows the effect of DCF concentration on the fluorescence emitted by platelets from normal and thalassemia donors. The platelets were either stimulated with H_2O_2 (2 mM) or left untreated. The fluorescence reached a plateau at 0.2 to 0.5 mM DCF, suggesting that within this range of DCF concentrations, the fluorescence intensity is not influenced by the intracellular DCF content. Further experiments were, therefore, performed with 0.4 mM DCF.

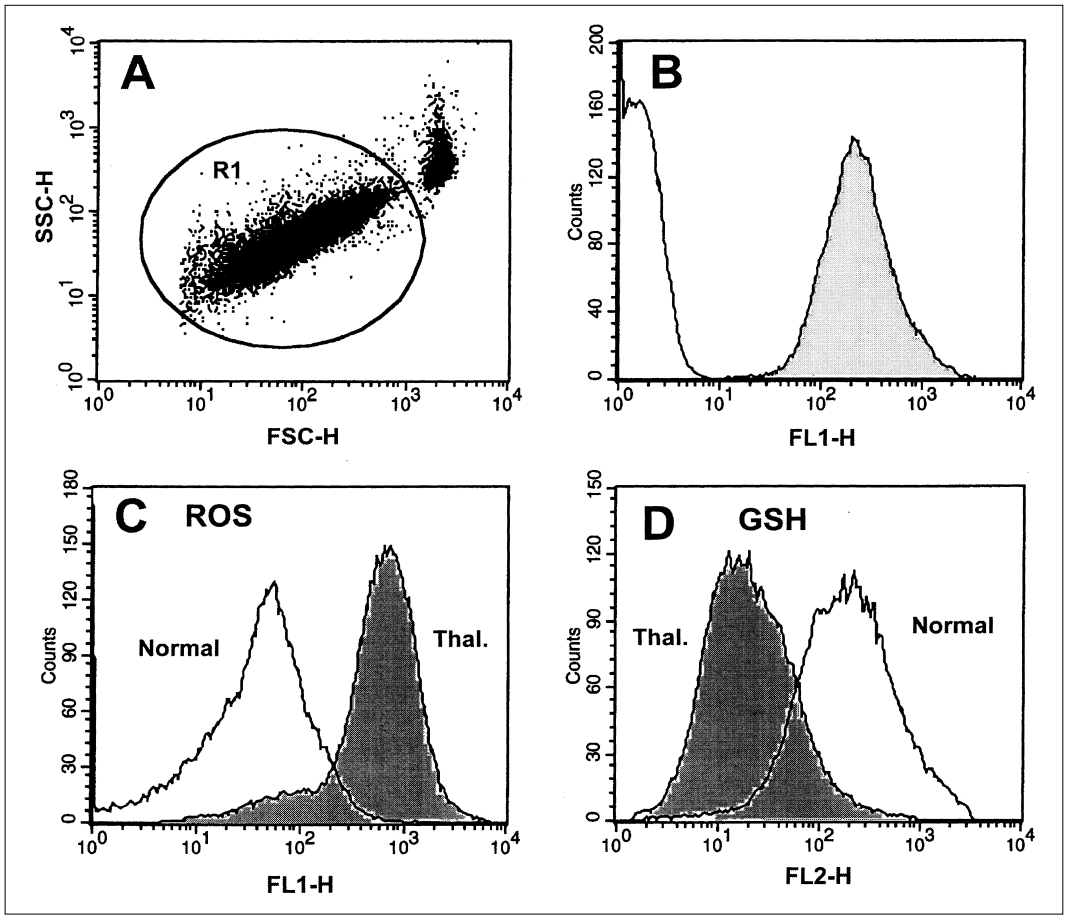

Figure 1: Flow cytometry analysis of ROS and GSH in platelets. Platelets obtained from a normal donor and from a patient with β-thalassemia major were stained with FITC-conjugated anti-CD41 antibodies or an isotype control. (A) A two-parameter dot-plot of forward light-scatter (FSC) and side light-scatter (SSC) of the normal sample. A gate (R1) was set around the platelet population. (B) Cells in R1 were analyzed for CD41. Histograms of unstained (white) and stained (light gray) samples are shown. The results show that all the cells in R1 were CD41 positive, namely platelets. Platelets from the thalassemia patient were also localized within the same gate (not shown). (C) For ROS measurement, platelets from normal and thalassemia donors were incubated with 0.4 mM DCF for 15 min at 37ºC, washed and then incubated for another 15 min at room temperature with 2 mM H_2O_2. (D) For GSH measurement, platelets from normal and thalassemia donors were incubated with 40 μM mercury orange for 3 min at room temperature. The platelets were then washed, suspended in PBS and analyzed by flow cytometry, as described in Materials and Methods. A gate (R1) was set on the FCS/SSC dot plot to exclude RBC and WBC. Frequency histograms of gated platelets from normal (white) and thalassemia donors (gray) with respect to DCF (FL1-H) (C) and mercury orange (FL2-H) (D) staining are shown. The MFC of the platelets from normal and thalassemic samples were 61 and 681, respectively, in C, and 294 and 29, respectively, in D.

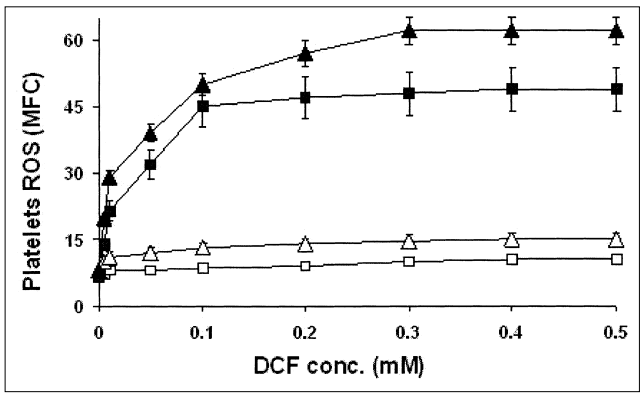

Figure 2: Effect of DCF concentration on ROS measurement. Platelets from normal (□, ■) or thalassemia (Δ, ▲) donors were treated for 15 min at room temperature with different concentrations of DCF, then washed and either stimulated (■, ▲) with H_2O_2 (2 mM) for 15 min or left untreated (□, Δ). Cellular fluorescence was measured by flow cytometry and the MFC (the mean± SD of 4 experiments) are presented.

To validate the use of mercury orange staining for measuring intracellular non-protein thiols in human platelets, thiol levels were modulated by treating platelets with NEM, which blocks total thiol groups, or with DEM, a specific non-protein thiol-depleting agent (18). The cells were then stained with mercury orange and cellular fluorescence was measured. The results demonstrate that incubation with NEM (Fig. 3A) or DEM (Fig. 3B) decreased the cellular fluorescence in a dose-dependent manner.

To test the relationship between platelet activation and platelet oxidative status, platelets from normal and thalassemic donors were labeled with DCF, washed and treated with thrombin (0.1 U/ml), calcium ionophore (5 μM) or PMA (400 ng/ml), compounds known to activate platelets (10), followed by measurement of their ROS content. All these agents stimulated ROS generation. Platelets from thalassemic patients were more responsive than platelets from normal controls (Fig. 4).

We analyzed ROS generation (Fig. 5A) and GSH levels (Fig. 5B) in platelets from more than 46 normal donors (with normal hemoglobin and hematocrit) and 46 samples from 22 patients with β-thalassemia. The MFC of the individual measurements are shown. The average MFC values of the platelets from the 46 normal donors and from the 22 thalassemic patients were 176 ± 99 and 314 ± 81, respectively, for ROS, and 319 ± 87 and 113 ± 47, respectively, for GSH. The differences between the normal and the thalassemic groups were highly significant ($p < 0.0001$, according to Student's t-test).

We previously showed that thalassemic RBC have higher ROS levels than do their normal counterparts (19). To determine whether thalassemic RBC affect platelet ROS, we exposed normal platelets for 3 hr at 37°C to heterologous RBC (of a compatible blood group) from thalassemic or normal donors. Platelets from normal donors incubated with their own RBC served as control. The platelets were then separated by centrifugation for 12 min at 800 rpm, washed, resuspended in PBS and their ROS were measured. The MFC was 2.5-fold higher in platelets incubated with thalassemic RBC than in platelets incubated with normal RBC (Fig. 6A). Platelets incubated under the same conditions, but in the absence of RBC, showed only a slight increase in ROS. These results demonstrate that thalassemic RBC induce a higher level of ROS in platelets than do normal RBC.

We next determined whether this effect of thalassemic RBC on platelets was related to oxidative damage to the RBC. Normal RBC were treated with 8 mM H_2O_2 for 3 hrs at room temperature or left untreated. We previously showed (19) that such treatment augmented generation of ROS by normal RBC 16-fold. The treated and untreated RBC samples were then incubated with their autologous platelets for 3 hrs at 37°C. Figure 6A shows that ROS generation in normal platelets incubated with their own H_2O_2-treated RBC was higher (MFC = 155 ± 29) than that in platelets incubated with their own, untreated, RBC (MFC = 48 ± 14).

To assess the effect of plasma on platelet ROS, normal platelets were incubated for 3 hrs at 37°C with plasma from thalassemia patients (of a compatible blood group) or with autologous plasma. After incubation, the platelets were spun down, washed and resuspended in PBS for ROS determination. The results show (Fig. 6B) that the ROS level was much higher (MFC = 698

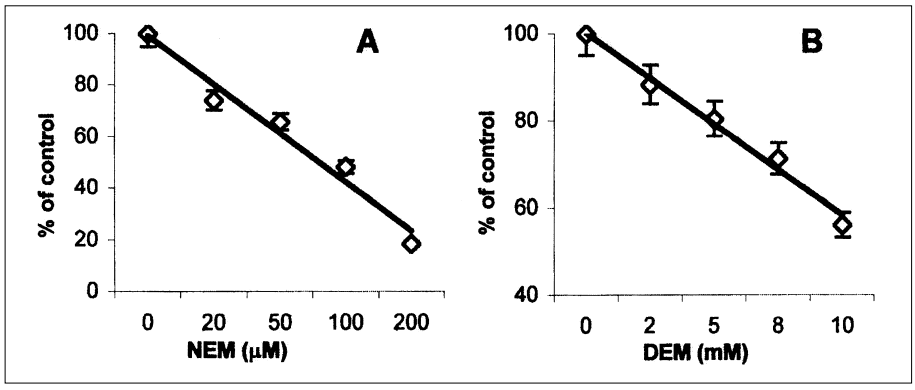

Figure 3: Effect of thiol-depleting agents on mercury orange staining. Platelets from normal donors were treated at room temperature with different concentrations of NEM (A) for 5 min or with DEM (B) for 4 hrs prior to staining with mercury orange. Cellular fluorescence was measured by flow cytometry and the MFC (the mean± SD of 4 experiments) are presented as the percentage of the control, untreated, platelets.

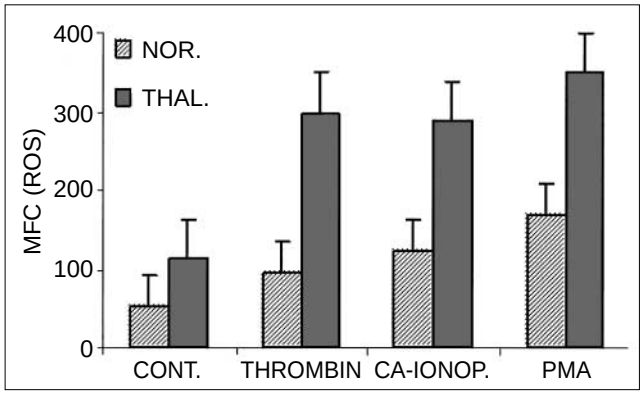

Figure 4: The effect of platelet activators on ROS generation. Platelets from normal and thalassemia donors were labeled with DCF, washed and treated for 30 min at room temperature with thrombin (0.1 U/ml), calcium ionophore (5 μM) and PMA (400 ng/ml) or left untreated (CONT.), followed by measurement of their ROS content. The results are presented as the mean± SD of 4 experiments.

± 42) in platelets incubated in plasma from thalassemia patients, than in platelets incubated in normal plasma (MFC = 562 ±24). Plasma contains various oxidants, including transferrin-bound and non-transferrin bound iron. When plasma of normal individuals and thalassemia patients was treated with the iron-chelator Desferoxamine (0.5 mM) (Fig. 6C) and incubated with normal platelets, the fluorescence of the platelets declined.

To further test the effects of oxidants and antioxidants on platelet ROS generation and GSH content, DCF- or mercury orange-stained platelets were treated for 15 min at 37°C with H_2O_2 (2 mM), BHP (0.5 mM), iron sources in the form of ferric ammonium citrate (0.2 mM) or hemin (heme chloride or heme arginate) (0.1 mM) or left untreated. The platelets were then washed, resuspended in PBS and treated for 15 min at room temperature with PBS or with the antioxidant NAC or vitamin C (both at 1 mM) (Fig. 7A, B). The results demonstrated that all the tested oxidants significantly increased ROS generation and decreased the GSH content. Subsequent to oxidant treatment, when platelets were treated with antioxidants, ROS and GSH returned to levels close to those of untreated cells.

Discussion

The ROS content in platelets has been measured by various techniques, using spectrophotometry (20), chemiluminescence, electron spin resonance and spin-trapping (21). We previously developed flow cytometric techniques for measuring ROS (19) and GSH in RBC (22). In the present study, we adapted these techniques for studying the oxidative status of platelets, measuring the fluorescence intensity of individual cells. This is a sensitive and rapid technique compared with standard biochemical assays. Although the data are expressed in arbitrary fluorescence units, the Mean Fluorescence Channel (MFC), rather than molar concentrations, it is useful for comparative purposes. Using a gating strategy, contaminating cells, such as neutrophils, with a high oxidative status, can be excluded from analysis without having to resort to their physical separation.

ROS was measured using the non-polar compound DCF which readily diffuses into cells. Upon crossing the membrane, it is deacetylated by intracellular esterases into a non-fluorescent polar derivative that is trapped inside the cells. Its oxidization by ROS produces a highly fluorescent derivative (23). The level of DCF fluorescence, reflecting the concentration of ROS, can be monitored by fluorescence spectrophotometry (20) or, as was done in the present study, by flow cytometry. Although the

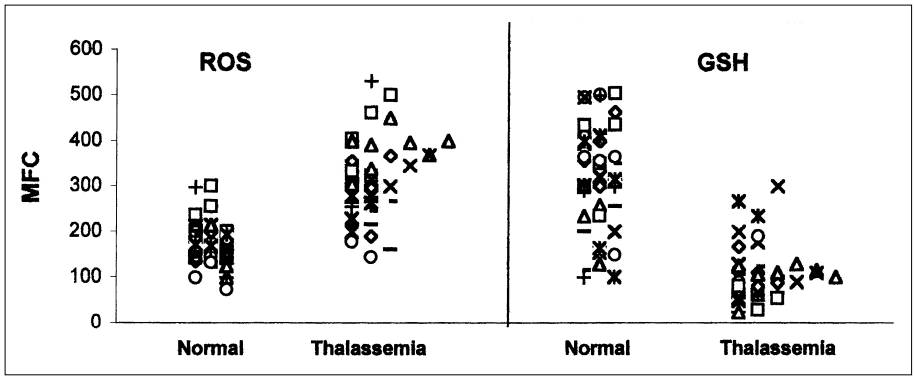

Figure 5: ROS generation and GSH content of platelets from normal and thalassemia donors. Forty-six blood samples from normal donors and 46 samples from 22 patients with β-thalassemia (9 patients donated 1 sample each, 7 patients - 2 samples, 4 patients - 3 samples, 1 patient – 5 samples and 1 patient donated 6 samples, at various times during a 4-month period. The platelets were analyzed for ROS and GSH content as described in *materials and methods*. The MFC of each sample is shown; samples of the same patient are represented by the same symbol. The average measurement of patients donating multiple samples was calculated (not shown). The mean MFC± SD of platelets from the 46 normal donors and 22 thalassemic donors was 176 ± 99 and 314 ± 81, respectively, for ROS generation, and 319 ± 87 and 113 ± 47, respectively, for GSH levels. The differences between the platelets from normal and thalassemia donors were highly significant ($p < 0.0001$, according to Student's t-test).

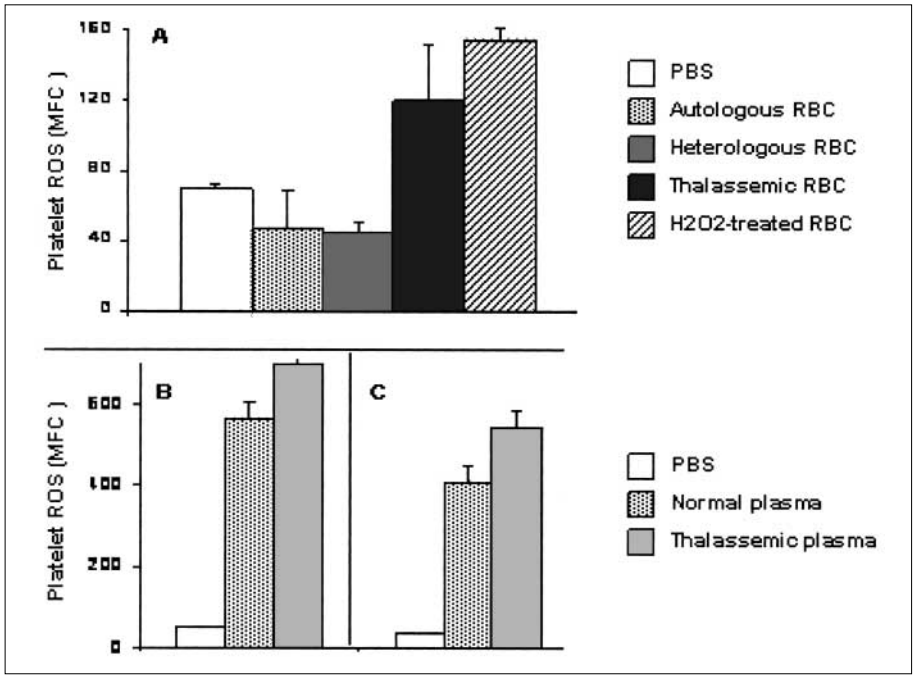

Figure 6: Influence of RBC and plasma from thalassemia patients on platelet ROS. (A) Platelets from normal donors were incubated for 3 hrs at 37ºC with thalassemic RBC (of an ABO compatible blood group), normal RBC of the same donor (autologous RBC) or different donors (heterologous RBC), or with their own RBC that had been treated for 3 hrs with 8 mM (H_2O_2-treated RBC). The platelets were then separated, by centrifugation for 12 min at 800 rpm, washed, resuspended in PBS and their ROS measured as described in Materials and Methods. Plasma from thalassemia patients, was untreated (B) or treated (C) with 0.5 mM Desferoxamin for 3 hrs at 37ºC and then incubated with normal platelets, followed by ROS measurement. The results were compared to ROS of normal platelets incubated with their own plasma or PBS. The results are presented as MFC of platelet ROS. The results are presented as the mean of 4 experiments ± SD.

DCF content of platelets under different conditions (e.g., in platelets derived from normal donors and thalassemic patients) may vary, dose-response analysis (Fig. 2) indicates that the DCF, at the concentration used (0.4 mM), is at saturation in terms of its effect on platelet fluorescence. The applicability of the method to measurement of platelet ROS was further validated by the increased fluorescence following treatment of platelets with the ROS-generating agents H_2O_2 (Figs. 2 and 7) and BHP (Fig. 7), and with the catalase inhibitor sodium azide (unpublished results), and the decreased fluorescence observed after treatment with the ROS scavenger NAC (Fig. 7). In addition, some experiments were repeated, and ROS was measured by dihydrorhodamine 123. This dye enters the cells freely, and after oxidation by ROS to rhodamine 123, emits a bright fluorescence signal that is exclusively localized inside the cell (24). Comparable results were obtained using both dyes (data not shown).

GSH was measured using mercury orange, which forms fluorescent adducts with GSH via the sulphydryl group, producing a S-glutathionyl derivative that emits red-orange fluorescence (16). The molecule reacts more rapidly with non-protein thiols, such as GSH, than with thiol proteins, allowing specificity under controlled staining conditions (13). Confirming the validity of this method in human platelets, NEM, which blocks total thiol groups (18), decreased the red-orange fluorescence of mercury orange in a dose-dependent manner (Fig. 3A). To ascertain that non-protein thiols were being stained under our staining conditions, we incubated platelets with DEM, a specific non-protein thiol-depleting agent. DEM is a weak electrophil of the α,β-unsaturated carbonyl group, which reacts with GSH only in the presence of glutathione transferase (18). DEM markedly suppressed the mercury orange fluorescence (Fig. 3B), suggesting that GSH was the principle thiol being stained by the dye. Although in the present work we have not confirmed that mercury orange is specific for GSH, the assay measures predominantly GSH, since it is the main non-protein thiol constituent of the thiol pool in platelets (25).

The results of the present study indicate that, compared with platelets from normal donors, platelets obtained from thalassemic patients produce more ROS and contain lower GSH levels, indicating a state of oxidative stress. Platelets have internal pathways for generating ROS (21, 26) although, in the absence of any known inherent abnormality in thalassemia platelets, it is reasonable to attribute their oxidative stress to continuous exposure to oxidative insults from extra-platelet sources.

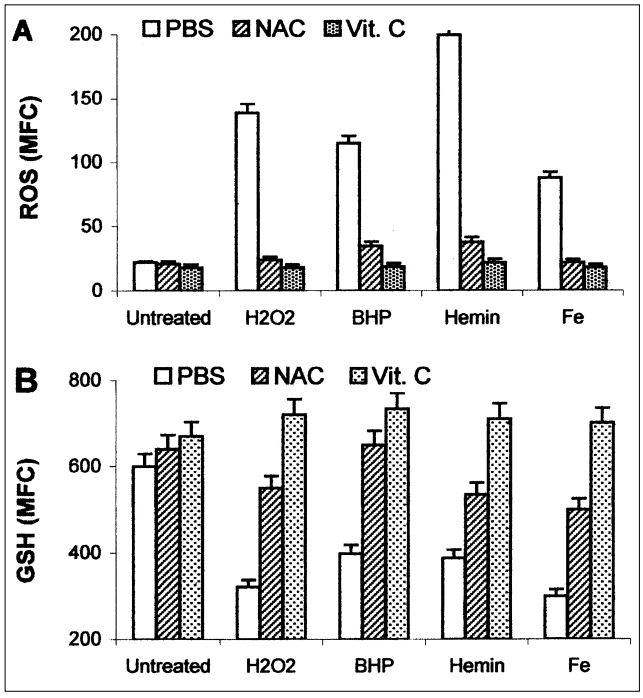

Figure 7: Effect of oxidants and antioxidants on platelets' ROS and GSH. Normal platelets stained with DCF or mercury orange were incubated for 15 min at 37ºC with or without H_2O_2 (2 mM), BHP (0.5 mM), hemin (0.1 mM) or ferric ammonium citrate (Fe) (0.2 mM). Each sample was then incubated with NAC (1 mM) or vitamin C (1 mM). The platelets were washed and their ROS (A) and GSH (B) measured. The results are presented as MFC of platelet ROS (the mean of 4 experiments ± SD).

We found that exposure of platelets to oxidants, such as H_2O_2 and BHP, stimulated their oxidative stress (Fig. 7). Moreover, incubation of normal platelets with plasma from thalassemia patients, rather than with normal plasma, produced a higher oxidative status. Among the various components in the plasma that may affect the platelets' oxidative status are iron-containing compounds such as transferrin-bound iron and non-transferrin-bound iron, ferritin, heme or hemoglobin, whose concentration is greater in thalassemia plasma (2, 8). Iron is involved in chemical reactions producing ROS (Fenton reaction) (12, 15). We now show that incubation of platelets in plasma-free physiological salt solution with iron (ferric ammonium citrate), heme (hemin or heme arginate) (Fig. 7) or hemoglobin (not shown), stimulated the platelets' oxidative stress. The involvement of iron-containing compounds in the plasma in the platelets' oxidative stress can also be inferred from experiments in which the effect of thalassemia plasma on platelet ROS was inhibited by the iron-chelator Desferoxamin (Fig. 6C).

The platelets' oxidative status could also have been affected by RBC: it was higher in normal platelets incubated with thalassemic RBC than in those incubated with normal RBC (Fig. 6A). However, when normal RBC were stimulated by H_2O_2, a treatment which results in an elevated oxidative status (19), they affected platelet ROS to a much greater extent than unstimulated RBC (Fig. 6A). These results suggest that thalassemic RBC, having a higher than normal ROS level, mediate oxidative stress in platelets directly, probably by contact or close proximity. Other studies indicated that platelets could be activated by ROS generated by other cells, such as neutrophils (17, 27). RBC might affect platelets also indirectly by a variety of mechanisms: release of iron-containing oxidants into the surrounding medium (plasma) (28, 29) as mentioned above. Release of ROS, such as superoxide anions, may cause oxidation of low-density lipoprotein (20) which, in turn, might activate platelets (30). Thalassemic RBC carry higher than normal exposed levels of phosphatidylserine moieties, which act as a procoagulant that amplifies the generation of thrombin and thus initiates platelet activation (31). Another indirect mechanism could be the release of hemin, iron or hemoglobin, which enhance platelet aggregation (28, 32).

It should be noted that most of the thalassemic patients studied were both splenectomized and on blood transfusion therapy. Blood transfusion did not affect the results of the thalassemia platelets, since in most cases the patients received packed RBC, largely devoid of platelets. In any event, circulating platelets have a short life-span, and since samples were withdrawn at least 3 weeks post transfusion, it is reasonable to assume that no donor platelets survived and all the measured platelets were of patient origin. If, indeed, platelets are affected by RBC, transfusion of RBC could have an indirect effect on the patients' platelets by diluting the thalassemic RBC, and thus ameliorating their adverse effect on the platelets' oxidative status.

Splenectomy could affect platelets quantitatively as well as qualitatively. Since the spleen scavenges old /damaged RBC and platelets, in the absence of a spleen, these cells remain in the circulation. Damaged RBC could stimulate oxidative stress in platelets, which accumulate in the blood in their activated state, thus, contributing to thromboembolic phenomena.

The effect of oxidizing agents on platelet ROS and GSH was inhibited by antioxidants such as NAC and vitamin C (Fig. 7). We previously found that both NAC (19) and vitamin C (unpublished observation) decreased ROS generation and increased the GSH level in thalassemic RBC. It has been shown that after crossing the membrane NAC is converted into cysteine, a precursor of reduced glutathione. Using a different methodology, Olas and Wachowicz (33) discovered that vitamin C acts as an antioxidant in pig platelets. In addition, both agents inhibit platelet aggregation *in vivo* (34, 35). As regards its potential beneficial influence on platelets, the oxidants may have a dual effect: a direct effect on the platelets and an indirect effect by reducing the oxidative status of other cells (e.g., RBC) that affect platelets. With regard to vitamin C, it has been shown that at low doses it may exert pro-oxidative effects (33, 36). Therefore, caution should be exercised when prescribing it, especially in iron-overload, as in thalassemic patients.

The present results indicate that in thalassemia, platelets are exposed to oxidative stress, which causes their chronic activation and may result in thromboembolic consequences. This situation may also prevail in other RBC anomalies, such as sickle cell anemia, Polycythemia Vera and Paroxysmal Nocturnal Hemoglobinuria, which are also associated with thromboembolic phenomena. Our findings raise the possibility of using antioxidants in addition to antithrombotic drugs as a prophylactic treatment in these diseases.

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